In vitro and *in vivo* toxicity evaluation of plant virus nanocarriers

Agnese Blandino[1*§], Chiara Lico[2*], Selene Baschieri[2], Lanfranco Barberini[3], Carlo Cirotto[3], Paolo Blasi[1,5], Luca Santi[4]

[1]Department of Pharmaceutical Sciences, Università degli Studi di Perugia, via del Liceo 1, 06123, Perugia Italy

[2]Laboratory of Biotechnology, Technical Unit Radiation Biology and Human Health, ENEA Casaccia Research Center, Via Anguillarese 301, 00123 S. Maria di Galeria, Rome, Italy

[3]Department of Chemistry, Biology, and Biotechnology, Università degli Studi di Perugia, via elce di sotto, 06123, Perugia Italy

[4]Department of Agriculture, Forests, Nature and Energy (DAFNE), Università della Tuscia, Via San Camillo de Lellis snc, 01100 Viterbo, Italy

[5]Correspondence should be addressed to Paolo Blasi; e-mail: paolo.blasi@unipg.it, phone +39 0755852057, fax: +39 0755855123.

* Agnese Blandino and Chiara Lico contributed equally to this work.

§ present address: Acraf S.p.A. – Via Vecchia del Pinocchio, 22 60131 Ancona - Italy

Abstract

The use of biological self-assembling materials and of plant virus nanoparticles (pVNPs) in particular, appears very intriguing because it ensures a great choice of symmetries and dimensions, easy chemical and biological engineering of both surface and/or internal cavity, easy, safe, and rapid production in plants. In this perspective, we started to define the safety profile of two structurally different plant viruses produced in *Nicotiana benthamiana* L. plants: the filamentous Potato virus X and the icosahedral Tomato bushy stunt virus. *In vitro* haemolysis assay was used to test the cytotoxic effects, which could arise by pVNPs interaction with cellular membranes, while early embryo assay was used to evaluate toxicity and teratogenicity *in vivo*. Data indicates that these structurally robust particles, still able to infect plants after incubation in serum up to 24 hours, have neither toxic nor teratogenic effects *in vitro* and *in vivo*. This work represents the first safety-focused characterization of pVNPs in view of their possible use as delivery systems.

Keywords: nanoparticles, plant virus nanoparticles, Potato virus X, Tomato bushy stunt virus, drug delivery, toxicity, teratogenicity.

Introduction

After more than twenty years of academic and industrial research, nanometer-sized carriers, although still in early stages, are becoming a reality in the biomedical field, especially in vaccinology, drug delivery, and diagnostic. Different nanoparticle-based therapeutic and diagnostic agents are under preclinical and clinical evaluation and some of them are already on the market [1]. The most exploited and investigated nanoparticles (NPs) are those based on organic (e.g., lipids, polymers) and inorganic (e.g., metal, metal oxide) materials [2, 3]. Nonetheless, due to the extremely complex features that an ideal nano-delivery system should possess to achieve clinical application, none of these carriers is immune from drawbacks (e.g. difficult large-scale manufacturing, poor long-term stability, *in vivo* toxicity) [4, 5].

In this scenario, the crosspollination between biotechnology, nanoscience, nanotechnology, pharmaceutics and biomedicine is shifting the attention to bioinspired and/or bioengineered nanocarriers [6]. Nanoparticles derived from natural macromolecules self-assembly are considered extremely promising. Among the various bio-building blocks, self-assembling proteins have unique features as they can form supramolecular structures giving rise to symmetrical nano-objects [7]. These so called 'protein cages' can be made of ferritin-like proteins, chaperons, heat shock proteins and, most notably, viral proteins [8, 9]. The majority of virus nanoparticles (VNPs) display a spherical or a rod shape, and can be surprisingly diverse in terms of symmetries, dimensions and structure related properties. Additional intriguing features include the inherent monodispersity and extreme flexibility in terms of engineering strategies, the latter allowing the effective modification of the exposed surfaces and/or the exploitation of the internal cavity for cargo storage. For these reasons, VNPs have been used since the dawn of nanotechnology and are objects of intense research in the fields of targeted delivery, vaccinology, and imaging [10]. In this context, plant VNPs (pVNPs) are attracting increasing attention. Several plant viruses have been exploited for biomedical applications and tested efficiently in animal models [11]. Plants, such as the *Solanacea Nicotiana benthamiana* L., relative of common tobacco, allow a convenient and easily scalable production of pVNPs that, when outside their specific plant host, become protein nanoshells unable to replicate.

In this study we have focused on two distinct pVNPs-based systems: Potato virus X (PVX), the type member of the genus *Potexvirus* (*Alphaflexiviridae* family), and Tomato bushy stunt virus

(TBSV), the prototype member of the genus *Tombusvirus* (*Tombusviridae* family). Both viruses have a monopartite single stranded, positive-sense RNA genome. PVX is a flexible filamentous virus of ~500 nm in length and 15 nm in diameter, constituted by approximately 1300 units of a unique coat protein (CP). TBSV is a spherical virus of about 30 nm in diameter with 180 copies of the CP self-assembled to form a T = 3 icosahedral symmetry.

Both systems have been extensively explored as nanocarriers and the most investigated application is drug targeting to tumor cells. CP genetic engineering can be used to arrange the covalent binding of exogenous molecules (chemical derivatization strategy), such as drugs, targeting moieties, or to display peptide sequences that bind selectively to a specific receptor overexpressed on cancer cells (genetic engineering strategy). For instance, PVX and TBSV CP have been engineered to display heterologous polypeptides at their N- or C-terminus [12- 14]. Regions generating external loops following CP folding have been used for the same purpose in Cowpea mosaic virus [15].

Peculiar virus features have allowed the development of 2 different methods of particle loading: reversal viral pore gating (also termed infusion) and *in vitro* controlled reassembly [11]. By choosing the adequate strategy, virtually all kind of drug can be loaded in the virus core.

Reversal viral pore gating rely on reversible conformation changes of capsid structure that generate pores. These pores allow the diffusion into the viral core of molecules that are entrapped upon the recovery of the native structure. This loading method is very efficient but is limited to molecules smaller than the pore size. It was estimated that about 900-1000 doxorubicin molecules could be encapsulated in a Red clover necrotic mosaic virus protein cage. This loading density (number of doxorubicin molecule/particle volume) is comparable to that of Doxil®, an approved liposomal doxorubicin formulation [16, 17].

In vitro controlled reassembly contemplate viral particle disassembling and reassembling *in vitro* in the presence of the material to be encapsulated. This method has allowed the encapsulation of larger molecules (e.g. nucleic acids, proteins, synthetic macromolecules) [18, 19] and nanoparticles [20].

Protein based NPs such as PVX and TBSV are considered ideal in terms of biocompatibility, because their biodegradability should prevent toxicity due to accumulation in the body (i.e. waste

excretion). Plant viruses have evolved to infect plant hosts, and to this aim have developed infection strategies totally different from those adopted by animal viruses. Due to the lack of specific receptors for recognition and entry into host cells they cannot infect human cells. Nonetheless, even if they behave in these systems as unreplicative and biologically safe nano-objects, a possible intrinsic toxicity cannot be underestimated.

This work represents a first step for the safety evaluation of PVX and TBSV in view of their forthcoming use as functionalized nanocarriers. *In vitro* haemolysis assay has been employed to test cytotoxic effects, which could arise by their interaction with cellular membranes, while early embryo assay (EEA) was used to evaluate toxicity and teratogenicity *in vivo*.

Materials and Methods

Production and molecular characterization of pVNPs

PVX and TBSV propagation in *Nicotiana benthamiana* L. plants such as extraction and purification from plant tissues were performed as previously described [12- 14]. Protein concentration (i.e. CP concentration) in each preparation was determined using the bicinchoninic acid protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and further verified through 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of several dilutions of purified pVNPs and known quantities of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) comparing the intensities of Coomassie-stained bands. Purity was verified through SDS-PAGE and silver staining. The viral yield was calculated referring to the grams of fresh leaves tissue used for the extraction.

Structural characterization of the particles

NPs were morphologically characterized by transmission electron microscopy (TEM; Philips EM 400 T microscope, Eindhoven, The Netherlands). A drop of the purified pVNP suspension was deposed on the surface of a 200 mesh formvar® coated copper grid (TAAB Laboratories Equipment Ltd., Aldermaston, UK) previously treated with a poly-l-lysine solution. After 3 min, the pVNPs suspension was drawn off and replaced with a drop of negative staining agent (2% w/v phosphotungstic acid, pH 6.5). After 2 min, the stain drop was drawn off and the sample dried at room temperature (r.t.). Carbon nanoparticles (CNPs) (Sigma Aldrich Co., Saint Louis, MO, USA) and single wall carbon nanotubes (SWCNTs) [21] were suspended under sonication in ultrapure water and ethanol, respectively, and placed on 200 mesh formvar® coated copper grids. The samples were dried at r.t. and observed without negative staining.

Particles size was evaluated by dynamic light scattering (DLS), using a Nicomp 380 autocorrelator (PSS Inc., Santa Barbara, CA, USA) equipped with a Coherent Innova 70-3 (Laser Innovation, Moorpark, CA, USA) argon ion laser and a Peltier device for temperature control. The scattered light was detected at 90° from the incident light. TBSV samples were prepared by

dispersing 250 µg of viral particles in 250 µL of acetate buffer (50 mM, pH 5.2) while CNPs (nominal particle size determined by TEM <50 nm; Sigma Aldrich Co., Saint Louis, MO, USA) were dispersed in ultrapure water. Analyses were performed at 20°C, for 15 min, in triplicate.

Incubation of pVNPs in murine serum and plants infection

Whole blood was collected from C57BL/6J mice (Harlan Laboratories, Inc. Indianapolis, IN, USA) and allowed to clot by incubation at r.t. 15 min. The clot was pelleted through centrifugation at 2000 x g for 10 min at 4°C and the overlying serum collected. pVNPs were diluted in serum at a concentration of 400 ng/µl and incubated at 37°C for 0.5, 2, 4 and 24 h. In control samples pVNPs were diluted and incubated with phosphate buffered saline (PBS; 10mM Na_2HPO_4, 10mM NaH_2PO_4, 150 mM NaCl, pH 7.2). At each time interval, an aliquot of pVNPs was collected and used to infect *N. benthamiana* L. plants, as described above (with 20, 2 or 0.2 µg/leaf). As additional control, plants were infected with 20 µg/leaf of pVNPs diluted in PBS or serum but not incubated at 37°C, to test if serum *per se* could hamper viral infectivity.
Plants were daily controlled to monitor the onset of infection symptoms.

Haemolysis assay

Human blood was drawn in S-monovettes (Sarstedt, Nümbrecht, Germany) containing EDTA-K_3 as anti-coagulant. Red blood cells (RBCs) were washed three times with 0.9% NaCl by centrifugation at 1200 x g, r.t., no brake for 10 min and then suspended in Dulbecco's PBS Ca^{2+} and Mg^{2+} Free (DPBS CMF) (1:10). Two hundred µl of the RDBs suspension (corresponding to about $9x10^7$ cells) were distributed in triplicate into Eppendorf tubes containing 800 µl of 10% triton X-100 (positive control), DPBS CMF (negative control) or DPBS CMF added with different quantities (10, 100 or 200 µg) of pVNPs. The tubes were incubated at 37°C and mixed by inversion every 30 min. After 6 h all the samples were centrifuged at 1200 x g, r.t., no brake, 10 min. The supernatant of each sample was transferred in clean tubes to which 1/10 of the volume of Drabkin's reagent (Sigma Chemical Co., St. Louis, MO, USA) was added before optical density reading at 540 nm ($O.D._{540}$). The mean of $O.D._{540}$ readings for each triplicate was obtained, and the percentage of hemolysis was calculated using the equation [(A-B)/(C-B)]x100,

where A is the absorbance of the pVNP-treated sample, B is the absorbance of the negative control (blood cells incubated in DPBS CMF) and C is the absorbance of the positive control (blood cells incubated in triton X-100).

Chick embryo conditioning and treatment (early embryo assay, EEA)

Fertilized eggs (Cerquaglia Farm, Marsciano, Italy) were incubated on their side with small end pointed slightly down at 38°C and 60% relative humidity in an egg incubator. After 16 h, corresponding to Hamilton and Hamburger (H & H) development stage 3 [22], eggshells were cleaned with ethanol 70% and transferred under a sterile hood for further manipulations.

Embryos were divided in eight groups, consisting of three negative controls (untreated, simply pierced with the syringe needleor treated with chicken saline, 0.75% w/v NaCl), three positive controls (treated with 20 μg/egg retinoic acid (Comifar, Novate Milanese, Milano, Italy) , 10 μg/egg CNPs , 10 μg/egg SWCNTs), and two groups treated with pVNPs at five different concentrations (1, 10, 100 ng/egg, and 1, 10 μg/egg). Plant virus NPs and CNPs were suspended in chicken saline. Due to the extremely poor water solubility of retinoic acid and strong hydrophobicity of native SWCNTs, in this case corn oil was used as vehicle instead of saline.

To allow 22 gauge needle penetration and injection, a small piece of eggshell (about 2 mm) was removed from the egg acute pole. One hundred μl were carefully injected in the yolk sac, introducing the whole length of the needle (4 cm) to achieve the central part of the yolk. After treatment, the pierce was closed with adhesive tape to avoid microbial contamination and water loss. Treated embryos, as well as control embryos, were incubated at 38°C and 60% relative humidity for additional 26 h.

Toxicity and teratogenicity evaluation

Twenty-six hours after treatment, eggshells were opened and embryos carefully evaluated to individuate non-fertile eggs, embryos died before treatment, embryos died after treatment, and viable embryos. Thereafter, the blastodiscs of all the embryos were carefully removed from the yolk surface, washed with saline, observed and photographed using a stereomicroscope (Leica

WILD M32 with WILD PLAN 1X ocular; Leica, Wetzlar, Germany) equipped with a digital camera, and finally fixed with a 4% w/v paraformaldehyde solution.

Embryos representative of each group were stained with haematoxylin (Merck, Rahway, NJ, USA) and eosin (Carlo Erba, Milano, Italy) and mounted on glass slides with coverslips.

Toxicity was evaluated by comparing with the negative control both the number of dead embryos (died after treatment) as well as the number of somites. Teratogenicity was evaluated by analysing, qualitatively and quantitatively, somites, vascular area, and neural tube deformities.

Statistical analysis

Chi-square statistical test was used to test the null hypothesis (i.e. no significant difference between the expected and observed result). In particular, the number of embryos survived to the different treatments and the number of embryos survived in the untreated group (negative control) were compared.

To determine if the mean number of somites and standard deviation of the treated and untreated embryos were significantly different from each other Student's t-test was applied ($P > 0.05$ gave a not significant statistical difference).

RESULTS AND DISCUSSION

Viral and carbon nanoparticle characterization

Nicotiana benthamiana L. [23] plants were grown in an hydroponic system in a containment greenhouse, with microbiologically controlled water supplemented with a three parts nutrient (Flora SeriesTM: FloraMicro, FloraGro and FloraBloom) following the instructions of the manufacturer (General Hydroponics). Light (16/8 h day/night cycle; daily light integral 3.9 moles/day; photosintetically active radiation 136 μmol/m^2/s), temperature (25°C) and relative humidity (84%) were controlled. Six to 8 weeks after germination, plants were infected with PVX or TBSV. Between 7 and 10 days post infection (d.p.i.) all leaves showing infection symptoms were harvested, weighted and processed for virus extraction and purification. Viruses usually need about 7 days to spread throughout the plant (systemically), inducing leave alterations such as mottling, severe distortion, stunting, chlorosis and mosaic (Figure 1a and 1d). At later stages (11-14 d.p.i.), infection progression leads to necrosis of the apical shoots and, finally, to plant death. Viruses were purified from systemically infected leaves and recovered with yields ranging from 0.2 to 0.6 or even 1 mg/g of fresh leaves tissue, with lower yields associated to PVX, more difficult to extract and purify due to its filamentous nature. To verify the quality of the purification, pVNPs were analysed through SDS-PAGE. After electrophoretic separation and silver staining of the gel a single band corresponding to the viral CP (approximate expected molecular masses: PVX, 25 kDa; TBSV, 41 kDa) was visible for both TBSV and PVX NPs (Figure 1c and 1f). Gel staining with Coomassie blue using different dilutions of a quantified reference protein was also performed to confirm the yield rate (data not shown).

Purified pVNPs were further characterized through electron microscopy. The TEM photomicrographs of TBSV showed morphologically homogenous particles (Figure 1b), with regular shape and average diameter of ~30 nm [14]. PVX photomicrographs evidenced a filamentous and flexuous structure less homogenous in size. As expected, beside whole particles of ~500 nm in length, 8-10% of shorter rods were also present [24, 25]. The bimodal distribution is not an artifact of purification but a specific product of PVX replication strategy [24]. Notably, all PVX NPs were consistent in the diameter size (15 nm) (Figure 1e).

Due to the particulate nature of TBSV and PVX, CNPs and SWCNTs of similar size and shape have been characterized and employed as positive controls in EEA. TEM confirmed that CNPs have spherical shape and a mean particle diameter of about 30-40 nm (Figure 2a), compatible

with the nominal size reported by the supplier (i.e. <50 nm). SWCNTs appeared to form aggregates (Figure 2b). This is due to the high lipophilicity of SWCNTs that are well dispersed as single particles in a limited number of solvents, such as dimethyl sulfoxide that, due to the incompatibility with the Formvar® film of the TEM grid, could not be used for sample preparation. SWCNTs were dispersed in ethanol with the aid of ultrasounds but because of the non-optimal solvent properties and solvent evaporation, aggregation took place.

DLS analysis of TBSV confirmed TEM observations, indicating that the mean hydrodynamic diameter (mhd) of the particles is of 33.5 ± 0.2 nm with a very narrow Gaussian distribution width (4.73 ± 0.05 nm) (Figure 3). DLS showed the presence in the CNPs suspensions of 2 populations: one with a mhd of 35.2 nm (~90%) and another with a mhd around 540 nm (~10%) (Figure 3).

PVX and SWCNTs, being filamentous, could not be analysed by DLS since this technique allows to consistently estimate the dimensions of spherical objects only. Even if some attempts to use this approach to size tubular particles have been reported, the results are not reliable as the estimated mhd does not correspond only to one filament dimension (length or diameter) but to a combination of the two values [26- 28]. PVX particles diameters were determined through electron microscopy and showed the expected sizes [25].

pVNPs serum stability

NPs intended for drug delivery must circulate in the blood for a sufficiently long period of time to accumulate at the target site and, in order to maintain efficacy after intravenous injection, the interaction with serum proteins must not induce their proteolysis/disassembly and drug release/unloading. For this reason, the stability in serum is a fundamental requisite.

A negative effect of pVNPs interaction with blood components has been suggested for Cowpea Mosaic Virus (CPMV) NPs that, following incubation with plasma and/or serum, were significantly inhibited in their plant infectivity [29].

To verify if plasma/serum components have negative effects on TBSV and PVX, the pVNPs were incubated at 37°C for 24 h with either mouse serum or PBS, and then used to inoculate *N. benthamiana* L. plants at three different dilutions, monitoring daily the onset of symptoms. Symptoms first appeared, in a dose dependent fashion, on plants infected with TBSV that is more aggressive than PVX. Plants inoculated with 20 µg/leaf showed first systemic symptoms at day 4

for TBSV and at day 6 for PVX. Plants infected with the lowest dose (0.2 μg/leaf) showed a delay in symptoms appearance (day 8 for TBSV and day 10 for PVX), although both pVNPs finally spread systemically in all plants (data not shown).

These results clearly indicated that both pVNPs are extremely robust and maintain their integrity as well as their ability to infect plants up to 24 h after of incubation in PBS or serum.

In vitro haemolysis assay

During blood stream circulation, NPs should not affect the integrity of cellular components, erythrocytes in particular. *In vitro* haemolysis assay is recommended by US Food and Drug Administration for excipients intended for intravenous administration (http://www.fda.gov/ohrms/dockets/98fr/2002d-0389-gdl0002.pdf).

An *in vitro* haemolysis assay was performed to determine any detrimental effect exerted by PVX and TBSV on erythrocytes. Haemolysis assays indicated that 10 μg of both PVX and TBSV had no effects on erythrocytes integrity. At the higher concentrations TBSV still behave as the negative control, while PVX appears to induce a slight and dose-dependent haemolysis rate (1.8 and 2.7 % for 100 and 200 μg, respectively). The reason of this different behaviour is probably ascribable at one of the many features that distinguish the two pVNPs (e.g., shape, surface charge, and dimensions). However, it must also be mentioned that this rate of haemolysis is far less the tolerated 5% threshold considered the critical safe haemolytic ratio for biomaterials according to ISO/TR 7406 [30, 31]. Moreover, this effect is observed in experimental conditions that are very different and 'extreme' in terms of NPs/erythrocytes ratio and of interaction duration with blood cell components compared to those generally reported in literature.

Toxicity and teratogenicity in chicken embryos

Chicken embryo has been used extensively in experimental embryology and development biology. It has been employed as animal model for toxicity and teratogenicity tests after the pioneering work of McLaughlin in 1963 [32- 35]. In the 1990s, two experimental protocols were developed: chick embryotoxicity screening test (CHEST) [36], to evaluate pharmacological agents, and EEA [37], to evaluate teratogen potential during the first week of embryonic development. Due to the large number of results collected from this animal model, chicken embryo is considered an excellent model for assessing potential toxicity and teratogenicity [38].

However, the reliability of the model depends on a series of critical factors all contemplated and carefully respected in the present study. In particular, (i) adequate negative and positive controls, (ii) use of a minimum of 10 eggs per dose (20 is considered ideal), and (iii) right volume and route of administration [38].

Figure 4 shows embryos' viability of about 90% in the negative control (untreated) and a mean number of somites around 14-15 as reported in the H & H developmental stage 11-12 [22]. The two additional negative controls (i.e. simply pierced and treated with chicken saline), included to validate the injection manoeuvre and the injected volume, had the same mortality and the same number of somites of the untreated group [39]. Retinoic acid injection produced a significant abortifacient activity ($P < 0.01$) and a significant delay in somitogenesis ($P < 0.01$), both indicators of toxicity (Figure 4) [40, 41], inducing malformations in 54% of the survived embryos [42]. Figure 5b shows a classical example of malformations induced by retinoic acid on the somites and the neural tube: two or more somites merge together and/or with the neural tube forming what is known as 'zig-zag neural tube' or 'zig-zag somites' [42]. CNPs and SWCNTs were chosen as additional positive controls for their similarities in shape and size with TBSV and PVX and for their known toxic activity [43- 46]. CNPs induced high toxicity and teratogenicity with 50% of mortality and malformations in all the viable embryos (i.e., abnormal somites morphology, open neural channel and lacerations of the area pellucida) (Figure 5c). SWCNTs induced toxicity in terms of mortality but not in terms of alteration of the somites number. A low frequency of malformations has been recorded but even though embryos presented the right number of somites, the bodies were generally smaller (Figure 5d) .

TBSV and PVX, once injected at doses ranging from 1 ng to 10 μg/embryo, did not show neither signs of toxicity nor signs of teratogenicity. The viability was sometimes slightly lower than 90% but no significant differences were observed with the negative controls ($P > 0.05$). The mean number of somites, a well-established indicator of toxicity, did not show significant statistical differences when compared to the negative controls (Figure 4). Malformations were not recorded in TBSV and PVX treated groups. Photomicrographs of embryos treated with TBSV (Figure 1s), reported as an example, showed size and general morphology comparable to that of the negative controls (Figure 5a). In fact, embryos at the H & H stage 11-12 (stage 11: 40-45 h, 13 somites; stage 12: 45-49 h, 16 somites) present the heart bent to right and slightly S-shaped and the forebrain covered by the headfold of amnion [47]. Higher magnification, together with

haematoxylin and eosin staining, allowed to prove a correct somitogenesis, the absence of fused somites and/or deformities of the neural channel (Figure 1s).

At the five concentrations evaluated, TBSV and PVX show neither toxicity nor teratogenicity on chicken embryos when compared to positive controls: retinoic acid, CNPs, and SWCNTs. In contrast to the absence of effects induced by the differently shaped pVNPs, a big difference is evident in the effects produced in terms of toxicity and teratogenicity by the differently shaped carbon nanostructures. CNPs appear to induce more pronounced damages, and this may be ascribed at first instance to characteristics related to their physico-chemical nature. In fact, CNPs were easily dispersed in an aqueous solvent (saline 0.75% w/v) while SWCNTs required the use of corn oil as organic solvent [48]. Even if SWCNTs seemed well dispersed in the oil, when explanting the blastodisk it was possible to observe black spots in the yolk indicating post-injection aggregation (data not shown). The shifting from nanomaterials (of definite size and morphology) to micrometric aggregates with lower surface-area-to-volume ratio, strongly changes the interactions with biological systems, including the possible reduction of the toxic potential.

CONCLUSIONS

The overall morphological and structural analysis of both pVNPs confirmed the reliability of these "objects" as carriers for drug delivery and the superiority of their production system (plants bio-factories) if compared to conventional ones.

The overall toxicological analysis indicate that TBSV and PVX are not haemolytic *in vitro*, nor teratogenic or toxic in the chicken embryo model. These data are fundamental for the correct and safe development of these carriers in the light of future clinical applications.

Finally, even if TBSV seems more versatile in this field of application thanks to its ability to load at its interior a drug cargo, the flexible filamentous shape of PVX could represent an added value in view of possible applications in nanomedicine due to their superior pharmacokinetic and tumour homing properties. In fact, in spite of the length heterogeneity, a problem that can be solved through further gradient purification steps, the use of filamentous nano-objects has been explored and seems to have different *in vivo* behaviour as compared to spherical particles.

Declaration of interests

The Authors declare no conflict of interest.

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